BIOENGINEERED SKIN FROM STEM CELLS FOR TREATMENT OF CUTANEOUS VESICANT INJURY

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ABSTRACT

Sulfur mustard is a chemical warfare agent and potent vesicant that penetrates rapidly through the skin and causes prolonged injuries and incapacitation. Severe exposure to HD induces blistering skin reactions and significant loss of stem cell keratinocytes that are required for a continuous renewal of the epidermal cell layer. Therefore, HD injuries require long healing periods leaving significant cosmetic and/or functional deficits. We are developing bioengineered skin from embryonic stem cells for improved therapy for HD-induced skin damage. Since stem cell keratinocytes lack major histocompatibility class II antigens, they exhibit little immunogenicity and are suitable for allograft use. The multipotential nature of stem cells may be particularly valuable for skin damage where several cell types and growth factors are necessary for proper repair and regeneration.

We differentiated embryonic stem cells to skin keratinocytes in a cellular matrix cultured in an air/liquid system to form the bioengineered skin, mimicking normal skin. The efficacy of bioengineered skin for HD-induced skin lesions was evaluated using a C57BL/6 mouse model and 2-chloroethylethyl sulfide (half-mustard or CEES). Mice were exposed to 3 µL neat CEES for 10 minutes, and at 48 h post exposure, the injured skin was excised and the site was cleaned with debridase. Next, the bioengineered skin was transferred directly to the wound and affixed with a non-adherent sterile gauze pad. The embryonic stem cell derived bioengineered skin exhibited growth and healing in 1 to 3 weeks. The edema decreased and skin contraction around the exposed area was minimal. In contrast, the CEES exposed animals not treated with bioengineered skin had a) no skin growth and b) obvious contraction of the skin in the injured area. We also evaluated cryo-technologies to preserve the bioengineered skin for immediate application in the battlefield and then assessed their efficacy in the CEES model. Our results demonstrate that for the first time, topically applied fresh or frozen bioengineered skin or skin keratinocytes from embryonic stem cells produce improved healing when applied 48 h after HD exposure.

1. INTRODUCTION

Sulfur mustard (2, 2'-dichloro diethyl sulfide, HD) is a powerful alkylating agent that has been used as a chemical warfare agent since World War I. Skin exposed to HD initially produces an inflammatory response that progresses to large blisters and necrosis in humans. Overt signs of injury are not apparent until 24 h later, although damage occurs immediately after exposure (Dacre and Goldman 1996; Smith and Skelton 2003; Smith et al. 1995; Smith and Dunn 1991). HD induced damage is due to alkylation and cross-linking complementary DNA strands, proteins, and other biological matrices resulting in DNA mutations, cellular energy depletion, and inhibition of cell division. Despite efforts during the last 50 years to find prophylactic or post–exposure therapies, only supportive treatments have been established to date.

Historically, medical treatment of HD-induced damage is blister aspiration and/or deroofing (epidermal removal) and debridement, followed by extended treatment with topical antibiotic and sterile dressings. More recently, laser debridement (lasablation) of the deep dermal HD lesions followed by autologous split-thickness skin grafting was found to be more efficacious in improving wound healing (Graham et al. 2002). However, HD injury results in significant loss of stem cell keratinocytes that are required for a continuous supply of epidermal layer cells and successful healing. Skin stem cell keratinocytes are also required to prevent scar formation.

Due to the requirement of stem cell keratinocytes for improved therapy to HD-induced skin damage and that no such product is readily available, we developed a novel bioengineered skin using embryonic stem cells. Since stem cells are commercially available from C57BL/6 black mice, we developed a dorsal model of cutaneous vesicant injury in C57BL/6 mice to evaluate the bioengineered skin to avoid immune responses and host graft rejection. However, stem cell keratinocytes should be ideal for allograft application because they lack major histocompatibility class II antigens and consequently less immunogenicity. The pluri-potential nature of embryonic stem cells may be particularly valuable for

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skin damage where several cell types and growth factors are necessary for proper repair and regeneration. We also evaluated cryopreserved bioengineered skin for immediate application during a terrorist attack or in the battlefield.

2. MATERIALS AND METHODS

2.1 Materials: Mouse embryonic stem cells (C57BL/6 origin) and mitotically arrested DR4 feeder fibroblasts were obtained from Open Biosystems (Huntsville, AL). Fetal bovine serum, D-MEM, sodium pyruvate, L-glutamine, penicillin/streptomycin mixture, and trypsin were all purchased from Quality Biological Inc. (Gaithersburg, MD). Leukemia inhibitory factor was purchased from Stem Cell Technologies (Vancouver, British Columbia). Six-well collagen I inserts for the culturing of bioengineered skin were purchased from BD Biosciences (San Jose, CA). Dispase was obtained from Becton-Dickinson (Bedford, MA). Ascorbic acid, 2-mercaptoethanol, non-essential amino acids, and CEES (2-chlorethyl ethyl sulfide) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphate buffered saline (PBS) was purchased from Biofluids Cell Culture Products (Rockville, MD). Antibodies to filaggrin (stratum corneum), cytokeratin-14 (stratum basal), fibronectin, and collagen type IV for immuno-fluorescence microscopy were purchased from Pharmingen (San Diego, CA). The sterile gauze pads were purchased from Hermitage Hospital Products (Niantic, CT). Isoflurane was purchased from Halocarbon Laboratories (River Edge, NJ).

2.2 Animals: Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Male C57BL/6 black mice, five weeks of age with initial weights of 25g, were purchased from Charles River Laboratory (Wilmington, MA). Mice were kept for a one week stabilization period before use. This study's protocol was approved by the Institutional Animal Care and Use Committee, Walter Reed Army Institute of Research, Silver Spring, MD 20910 (protocol # B02-05).

2.3 Embryonic stem cell culture and preparation of bioengineered skin: Mitotically-arrested mouse embryonic DR4 feeder fibroblast (MEF) cells were cultured in stem cell culture medium for four days. Pluripotent mouse embryonic stem (ES) cells were then seeded on the DR4 feeder fibroblasts and cultured with leukemia inhibitor factor that prevented cell differentiation. Embryonic stem cells were trypsinized and frozen according to the instructions of the supplier. To prepare bioengineered skin, DR4 feeder fibroblasts were plated in 6 well Costar plates for 4-7 days and then trypsinized to remove the cells. Embryonic stem cells were seeded on the cellular matrix left behind after trypsinization. The embryonic stem cells were differentiated to skin keratinocytes by the addition of ascorbic acid (Coraux et al. 2003; Aberdam 2004). After two weeks, the cells were dislodged with dispase treatment and seeded on the insert membrane of a biocoat cell culture plate coated with a cellular matrix. Growth medium containing 0.3 mM ascorbic acid was added to cells from the top and bottom compartment. After 2-3 weeks, the bioengineered skin layer became thick and uniform; the medium was removed from the upper compartment and exposed to air. The bioengineered skin layer was cultured in the air/liquid culture for one week by feeding from the bottom compartment. The formation of the bioengineered skin was verified by immunofluorescence microscopy using dermal layer specific marker antibodies (Nambiar et al. 2004). Antibodies to filaggrin were used to detect stratum corneum, cytokeratin-14 was used for stratum basal, and fibronectin and collagen type IV was used to determine the basal layer.

2.4 Evaluation of the therapeutic efficacy of the bioengineered skin in the C57BL/6 dorsal model of cutaneous vesicant injury: The C57BL/6 black mouse dorsal model for cutaneous vesicant injury by 2-chloroethyl-ethyl-sulfide (half-mustard, CEES) was used as follows: 3 µl neat CEES was applied to the dorsal side of mice for 10 min followed by decontamination and recovery for various time intervals (24, 48, 72 and 96 h). First, animals were anesthetized using 1-5% isoflurane gas (O_2 flow rate 0.2 L/min) in a Viking Medical animal anesthesia chamber (Medford Lakes, NJ). The anesthetized mice were then transferred to stereotaxic equipment (David Kopf Instruments, Tujunga, CA) in an approved chemical safety hood for CEES application. The animals were maintained under anesthesia on the stereotaxic equipment, dorsal side up, and then shaved with a small Oster Golden A5 animal clipper (McMinnville, TN), being careful not to injure the skin. A vertical line was drawn from the top to the bottom of the dorsal region to keep the CEES exposed areas separate. Two circles (7 mm diameter) were drawn on each side of the vertical line (3 mm from line) in order to apply CEES precisely. Second, the center of each circle on the back was exposed to 3 µl of neat CEES using a 10 µl pipetter, and then covered with a small plastic lid to prevent evaporation. Third, after a 10 min exposure to CEES, the site was decontaminated with a proprietary decontamination solution. The CEES exposed site was decontaminated three times using fresh pieces of sterile gauze; the gauze was dipped into the decontamination solution using forceps and gently applied to the injury site using circular motions. This process was also used to dry the exposed

site after cleaning. The mice were returned to their cages and evaluated at 24, 48, 72, or 96 h.

2.5 Decontamination solution: Decontamination solution was freshly prepared before use. The proprietary solution chemically reacts with free CEES and extracts it. Otherwise, it had no observable irritant effects on skin.

2.6. Treatment with bioengineered skin: In some cases, at 48 h post exposure to CEES, debridase (Mediwound, Israel) was applied to the injury site for 2 h to peel of the injured skin. In other cases, the dead skin of the injured area was tangentially dislodged with a sterile scalpel and the bioengineered skin was applied. The mice were properly bandaged and returned to their cages. The mice were monitored until they regained their ability to maintain sternal recumbancy. Over the next two weeks, the mice were monitored daily and photographed to document healing.

2.7 Edema and histopathology analysis: Edema was quantified by determining the wet/dry weight ratio of the skin biopsy of the animals exposed to CEES and treated with either bioengineered skin or saline. Briefly, at different time points after bioengineered skin treatment, the mouse was euthanized in a carbon dioxide filled chamber (1 liter/min) followed by cervical dislocation. The CEES + saline or CEES + bioengineered skin treated areas were excised using a sterile blade, scissors, and forceps. Half of the skin sample was used for edema determination and the other half for histopathology. The extent of edema was evaluated by measuring the wet samples in a pre-weighted tin foil. The biopsies were then placed in an oven at 100 °C for one week, and then the dry weight of each sample recorded. The wet/dry ratio was used as a measure of edema. Samples were prepared for histopathological evaluation in a solution of 4% paraformaldehyde in phosphate buffered saline (PBS). They were then processed for paraffin embedding. Sections were serially cut and stained with hematoxylin and eosin for light microscopy using an Olympus (Center Valley, PA) microscope, and evaluated by a board certified veterinary pathologist.

2.8 Cryopreservation of the bioengineered skin: Five different cryopreservation solutions were prepared under sterile conditions (Table 1) by filtration through an Acrodisc® 0.2µm sterile disposable filter assembly attached to a 10ml syringe. Bioengineered skin after approximately 4 weeks of culturing were carefully removed from the original culturing plates using sterile forceps and placed into new sterile culture dish. The skin along with the attached membrane filter was removed using a sterile scalpel and divided into four equal quadrants. Each quadrant was placed in 500 µl of the different cryopreservation solutions in 2 ml cryo-preservation vials, which were placed into a -80ºC freezer overnight. The following day, the samples were transferred to a liquid nitrogen freezer and preserved for one month prior to evaluation.

Table 1

Different cryopreservation solutions used for preserving bioengineered skin developed from mouse embryonic stem cells:

Number	Composition/method
1	A vitrification solution consisting of 3.58 M ethylene glycol and 2.82 M DMSO in PBS (Silvestre et al. 2002a)
2	A rapid freezing medium consisting of 2.25M ethylene glycol and 2.25 M DMSO prepared in S-PBS (Silvestre et al. 2002b)
3.	A medium containing 0.25 M sucrose, D-MEM and Ham F-12 supplemented with 10% glycerin (Yanaga et al. 2001)
4.	A medium containing 10% DMSO and 15% FBS in D-MEM
5.	A medium made up of D-MEM supplemented with 10% DMSO and 20% FBS (Kubo and Kuroyanagi 2005)

2.9 Assessment of cell viability of cryopreserved bioengineered skin: The cryopreserved bioengineered skin was thawed and cultured in stem cell culture medium (see section 2.3). The cells were re-suspended by pipetting and the cell viability was assessed by Trypan blue exclusion.

2.10 Evaluation of the therapeutic efficacy of cryopreserved bioengineered skin: Cryopreserved bioengineered skin was evaluated in the C57BL/6 dorsal model of cutaneous vesicant injury using CEES as described above.

2.11 Statistical analysis: The data was analyzed using Minitab Version 14. The p-values were determined using the Mann-Whitney test; p-values less than 0.05 were considered significant.

3. RESULTS

3.1 Embryonic stem cell culture and development of bioengineered skin: Mouse embryonic stem cells were cultured with mouse fibroblast feeder cells (in medium containing leukemia inhibitory factor that prevented differentiation) for up to 2 weeks (Fig. 1). Next, the emb-

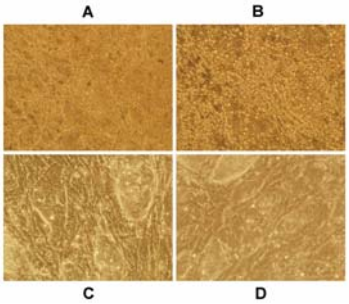

Figure 1: Pluripotent embryonic stem cells: C57BL/6 mouse embryonic stems cells were cultured in stem cell medium containing leukemia inhibitory factor. Photomicrographs were taken on day 4, 7 and 14 using an Olympus IX51 microscope. After day 14 the cells were frozen and used to grow the bioengineered skin.

ryonic stem cells were differentiated to skin keratinocytes on a cellular matrix by the addition of ascorbic acid, a free radical scavenger, keratinocytes survival factor, and bone morphogenic protein-4 (BMP-4). The cultures were then grown in an air/liquid medium to form the bioengineered skin. The formation of bioengineered skin was confirmed by immunofluorescence microscopy with antibodies to protein markers in skin including filaggrin (stratum corneum), cytokeratin-14 (stratum basal), fibronectin, and collagen type IV (Fig. 2).

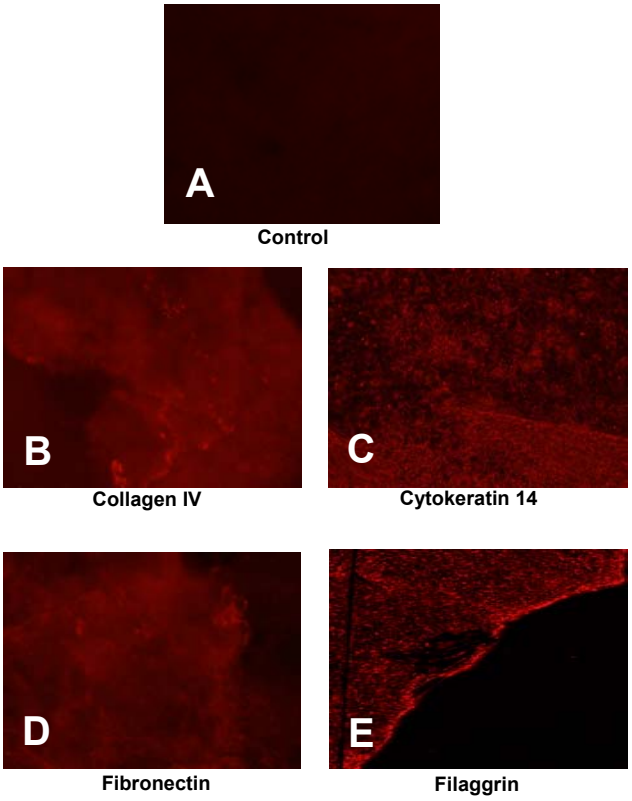

Figure 2: Immunofluorescence microscopy analysis of the bioengineered skin: The bioengineered skin was fixed in paraformaldehyde and immunolabelled with antibodies to specific skin markers as described in materials and methods. The immunostained slides were observed using an Olympus fluorescence microscope. A) Control secondary antibody labeled samples, B) collagen IV for basement membrane, C) cytokeratin14 for basal layer, D) fibronectin for suprabasal layer, and E) filaggrin for stratum granulosum.

3.2 C57BL/6 mouse dorsal model of cutaneous vesicant injury using CEES: The mouse skin appeared normal with no signs of injury after the 10 min exposure to 3 µl CEES followed by decontamination. However, 6 h after 3 µl CEES application, considerable injury with moderate erythema was observed. The skin injury was severe at 24 h with prominent red discoloration of the injured area. Swelling and protrusion of the skin was marked at 24 h and was maintained to 48 h (Fig. 3A and B). At later time points, there was less swelling and erythema. Eventually, 7-9 days after the onset of CEES exposure, the CEES exposed skin dried and exfoliated.

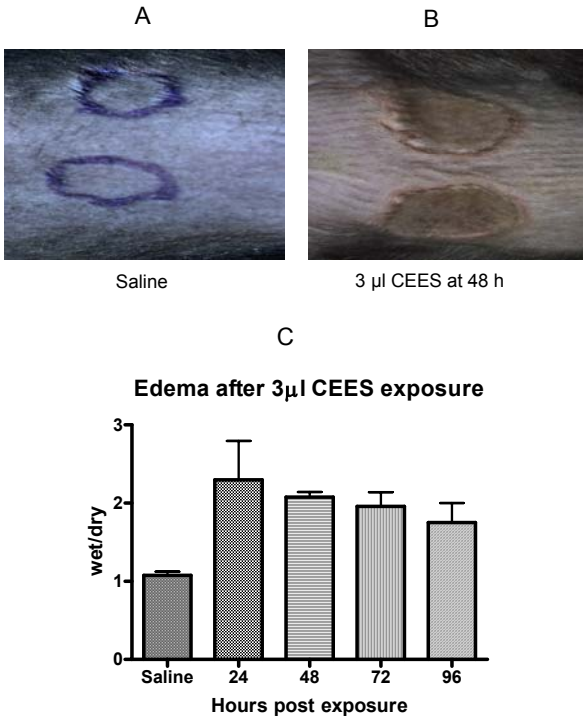

Figure 3: C57BL/6 black mouse dorsal model of cutaneous vesicant injury using CEES: Mice were exposed to A) saline or B) 3 µl of neat CEES for 10 min, decontaminated, and evaluated at 48 h post exposure. The animals were anesthetized and the injured skin was photographed using a Fuji Film S3 digital camera. The skin from a representative mouse exposed to CEES showed erythema and swelling. C) Mice exposed to 3 µl CEES and decontaminated after 10 min. At the indicated times, the mice were euthanized and the injured skin dissected and the wet/dry weight ratio was determined. The data is expressed as ratio (±) SEM. At 24, 48 and 72 h post exposure, edema was found to be significant ($p = 0.0142$, $n = 4$).

Edema was quantified by determining the wet/dry weight ratio of the skin biopsy of mice exposed to 3 µl CEES. Compared to saline controls, CEES exposure caused edema of the skin (Fig. 3C); a significant increase in the edema was observed at 24 h ($p = 0.014$, $n=4$). Further increase in the post-exposure time reduced the level of edema. Based on these findings we used 10 min exposure to 3 µl CEES and a time point of 48 h post exposure to evaluate the therapeutic efficacy of our bioengineered skin.

3.3 Evaluation of the therapeutic efficacy of bioengineered skin: To evaluate our bioengineered skin as a therapeutic for cutaneous vesicant injury, mice were exposed to 3 µL CEES for 10 minutes, decontaminated and then 48 h later the injured skin was excised and the site cleaned with debridase (Mediwound, Israel). Application of debridase for 2 h over the injured area did not aid in the removal of the CEES injured skin and was contraindicated due to the additional proteolytic activity. Therefore in later experiments, the CEES injured skin was removed at 48 h by excision and bioengineered skin was transferred directly to the wound and maintained for 10

min to permit adherence to the injured area before affixing with a non-adherent sterile gauze pad.

Two days after transplantation of the bioengineered skin, the injured area presented red color without contraction of the skin. Untreated CEES control animals showed initiation of skin contraction without healing. The skin contraction of the CEES control animal was clear at 4 days post exposure (Fig 4 A-right) compared to bioengineered skin treated animals (left). Seven days after treatment, bioengineered skin treated animals showed healing and skin growth, whereas the control CEES animals showed contraction of the skin and closing of the wound (Fig 4A). Hair growth at the injured area in the CEES controls was an indication of contraction of the skin rather than healing. Bioengineered skin treated animals showed an area of skin growth.

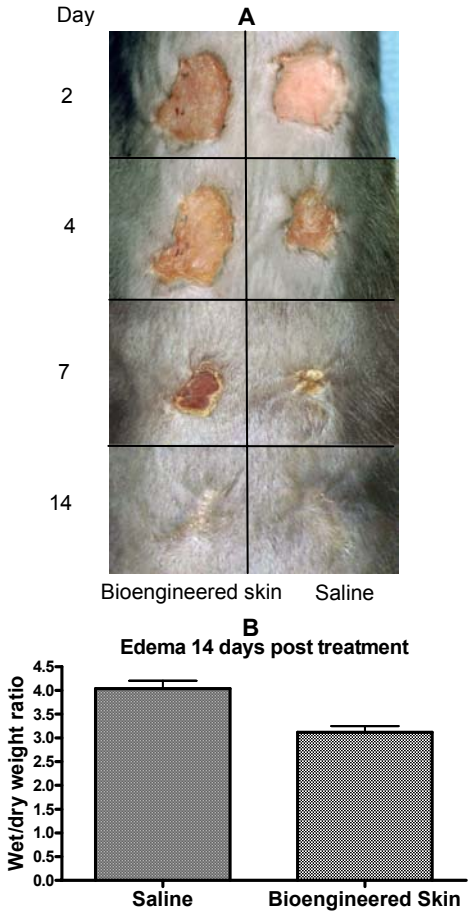

Figure 4: Evaluation of the therapeutic efficacy of the bioengineered skin in the CEES back model: A) Each side of the mice was exposed to 3 µl of CEES for 10 min and decontaminated. The left side of the animal was treated with the bioengineered skin and the right side was treated with saline at 48 h post exposure. The animals were anesthetized and the skin was photographed using a Fuji Film S3 digital camera at 2, 4, 7, and 14 days post-treatment. Treatment with bioengineered skin showed gradual healing with less contraction. On the other hand, CEES exposed animals treated with saline showed contraction of the skin. B) Skin edema of CEES exposed animals treated with bioengineered skin or saline at 14 days after the treatment. A significant difference was observed between the edema in mice treated with fresh bioengineered skin and mice treated with saline ($p = 0.0004$, $n = 23$).

3.4 Edema and histopathology analysis after treatment with the bioengineered skin: Compared to CEES exposed skin area without bioengineered skin treatment, bioengineered skin treated animals showed a significant reduction in the edema at 14 days (Fig 4B). Histopathological evaluation of the hematoxylin and eosin stained samples of bioengineered skin-treated mice showed significant epidermal growth and formation of epidermis (Fig. 5 A and B). Where as CEES controls showed epidermal necrosis combined with edema and an

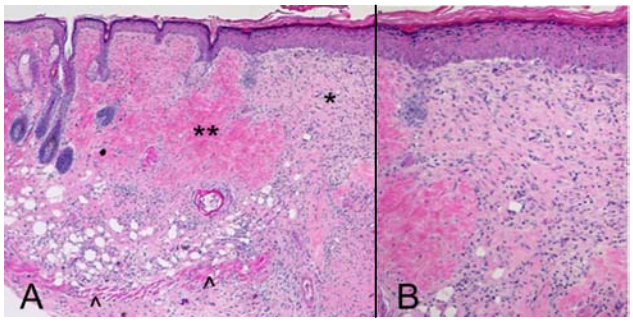

Figure 5: Histological analysis of CEES exposed animals treated with bioengineered skin: Two weeks after the treatment with the bioengineered skin, the animals were euthanized and the samples were processed for histological analysis. The sections were stained by hematoxylin and eosin and analyzed under a microscope. A. The dorsal skin from a mouse treated with saline after 3 µl CEES exposure showed necrosis of the epidermis (**), along with infiltration of inflammatory cells (arrowheads). B. The animals treated with bioengineered skin showed epidermal growth and healing.

infiltration of neutrophils in the dermis. Edema and neutrophilic infiltration extended into the panniculus and even the subcutaneous connective tissue, furthermore, minimal to mild hemorrhage was often present in edematous areas. The epidermal growth over the injury site in samples treated with bioengineered skin may now heal the underlying damage more rapidly.

3.5 Cryopreservation of the bioengineered skin and evaluation of therapeutic efficacy: Bioengineered skin cryopreserved in five different solutions (Table 1) was assayed for cell viability. The data showed a greater than 50% cell survival rate of the bioengineered skin cells in the medium containing 0.25 M sucrose, D-MEM and HAM-F12 supplemented with 10% glycerin (Fig. 6A, solution 3). Other solutions showed lower cell viability. Treatment using bioengineered skin cryopreserved in solution 3 on the C57BL/6 mouse model of CEES injury also showed lowest edema. Interestingly, cryopreserved bioengineered skin showed better cell growth compared to unfrozen bioengineered skin (Fig. 6B).

4. DISCUSSION

The major finding we present is that a) bioengineered skin can be developed from embryonic stem cells and b) the

bioengineered skin therapy improves wound healing following exposure to CEES (half mustard) in C57BL/6 black mouse model. Differentiation of embryonic stem cells and development of skin using an acellular matrix derived from fibroblasts was previously demonstrated (Coraux et al. 2003;Aberdam 2004) and we used an acellular matrix derived from fibroblasts of C57BL/6 mouse to treat CEES exposed mice.

Our data show that dorsal cutaneous exposure to 3 µl of the vesicant CEES for 10 min and decontamination followed by 48 h recovery is a reproducible model for the evaluation of bioengineered skin. A mouse ear

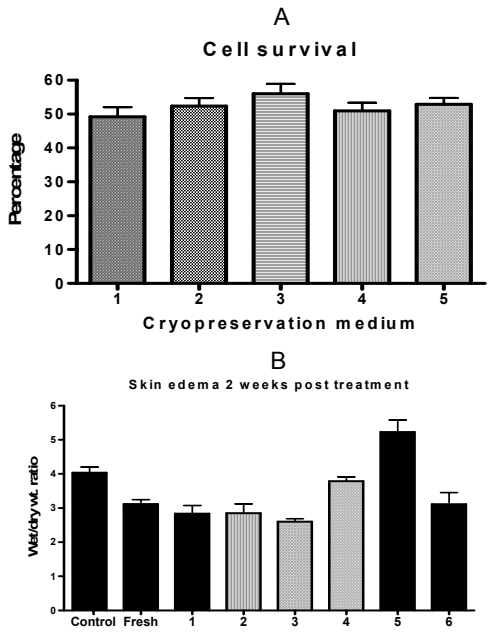

Figure 6: Evaluation of the viability and therapeutic efficacy of cryopreserved bioengineered skin in the C57BL mouse model of cutaneous vesicant injury: A) Bioengineered skin developed from embryonic stem cells were cryopreserved in 1) A vitrification solution consisting of 3.58 M ethylene glycol and 2.82 M DMSO in PBS 2) A rapid freezing medium consisting of 2.25 M ethylene glycol and 2.25 M DMSO prepared in S-PBS 3) A medium containing 0.25 M sucrose, D-MEM and Ham F-12 supplemented with 10% glycerin 4) A medium containing 10% DMSO and 15% FBS in D-MEM and 5) A medium made up of D-MEM supplemented with 10% DMSO and 20% FBS, overnight at -80°C and then in the liquid nitrogen for one month. The cells were thawed and re-suspended in stem cell culture medium. The viability of the cells was determined by Trypan blue exclusion. B) Skin edema of CEES exposed animals treated with cryopreserved bioengineered skin or saline at 14 days post treatment.

vesicant model using CEES and bioengineered skin resulted in exfoliated tissue with the ear skin. Evaluation of the bioengineered skin in the dorsal model clearly showed epidermal growth, healing, and less contraction compared to the untreated animals. Therefore, bioengineered skin is likely appropriate for all but mouse ear treatment.

It has been reported that laser debridement of the vesicant injured skin improves the healing. Debridement with debridase, a proteolytic enzyme, also improved HD skin injury in animal models (Dachir et al. 2004). However, we found that application of debridase at 48 h after CEES exposure did not aid in peeling of the exfoliating skin. Rather debridase increase the severity of the CEES injury, probably due to the additional proteolytic activity. Therefore, bioengineered skin was evaluated after excision of the injured skin in the dorsal model in the absence of debridase.

Transplanted bioengineered skin attached and adhered to the injured area. However, a larger size would likely be required to completely cover an area. In human exposures, an embryonic stem cell derived keratinocyte cell suspension could spread evenly and adhere to wounds to provide a uniform healing.

Treatment with frozen bioengineered skin enhanced protection compared to unfrozen bioengineered skin. More studies are required to identify the mechanism of improved protection by frozen bioengineered skin.

In summary, sulfur mustard (HD) is a potent cutaneous vesicant that penetrates rapidly through the skin, causing prolonged injuries and leading to severe incapacitation. To date, there are no rapid treatments available for HD-induced skin injuries, although there has been a long and intensive effort to find a therapeutic for HD skin lesions. Our results demonstrate for the first time, that topically applied bioengineered skin or skin keratinocytes from embryonic stem cells produce beneficial healing when applied 48 h after HD exposure in a mouse model. This technology offers a potential new treatment regimen for troops in the field exposed to chemical warfare vesicants.

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